

APPLICATION FOR  
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UNITED STATES PATENT AND TRADEMARK OFFICE

(Case No. HYZ-030CIP)

Title:

A METHOD OF DOWN-REGULATING GENE EXPRESSION

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# A METHOD OF DOWN-REGULATING GENE EXPRESSION

## CROSS-REFERENCE TO RELATED APPLICATIONS

5 This application is a continuation-in-part of  
cpendg Patent Application Serial No.  
08/709,910, filed September 10, 1996, which is a  
continuation-in-part of cpendg Patent  
Application Serial No. 08/328,520, filed October  
10 25, 1994.

## BACKGROUND OF THE INVENTION

15 The present invention relates to the control  
of gene expression. More particularly, this  
invention relates to the use of synthetic,  
modified oligonucleotides to down-regulate the  
expression of a gene in an animal.

20 The potential for the development of an  
antisense oligonucleotide therapeutic approach was  
first suggested in three articles published in  
1977 and 1978. Paterson et al. (*Proc. Natl. Acad. Sci.*  
(USA) (1977) 74:4370-4374) discloses that cell-free  
25 translation of mRNA can be inhibited by the  
binding of an oligonucleotide complementary to the  
mRNA. Zamecnik et al. (*Proc. Natl. Acad. Sci. (USA)*  
(1978) 75:280-284 and 285-288) discloses that a  
13mer synthetic oligonucleotide that is  
30 complementary to a part of the Rous sarcoma virus  
(RSV) genome inhibits RSV replication in infected  
chicken fibroblasts and inhibits RSV-mediated  
transformation of primary chick fibroblasts into  
malignant sarcoma cells.

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linkages have been shown to be more effective than their unmodified counterparts. For example, Agrawal et al. (*Proc. Natl. Acad. Sci. (USA)* (1988)

5 85:7079-7083) teaches that oligonucleotide phosphorothioates and certain oligonucleotide phosphoramidates are more effective at inhibiting HIV-1 than conventional phosphodiester-linked oligodeoxynucleotides. Agrawal et al. (*Proc. Natl. Acad. Sci. (USA)* (1989) 86:7790-7794) discloses the  
10 advantage of oligonucleotide phosphorothioates in inhibiting HIV-1 in early and chronically infected cells.

In addition, chimeric oligonucleotides having  
15 more than one type of internucleotide linkage within the oligonucleotide have been developed. Pederson et al. (U.S. Patent Nos. 5,149,797 and 5,220,007 discloses chimeric oligonucleotides having an oligonucleotide phosphodiester or  
20 oligonucleotide phosphorothioate core sequence flanked by nucleotide methylphosphonates or phosphoramidates. Furdon et al. (*Nucleic Acids Res.* (1989) 17:9193-9204) discloses chimeric oligonucleotides having regions of oligonucleotide  
25 phosphodiester in addition to either oligonucleotide phosphorothioate or methylphosphonate regions. Quartin et al. (*Nucleic Acids Res.* (1989) 17:7523-7562) discloses chimeric oligonucleotides having regions of oligonucleotide  
30 phosphodiester and oligonucleotide methylphosphonates. Inoue et al. (*FEBS Lett.* (1987) 215:237-250) discloses chimeric oligonucleotides

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having regions of deoxyribonucleotides and 2'-O-methyl-ribonucleotides.

5 Many of these modified oligonucleotides have contributed to improving the potential efficacy of the antisense oligonucleotide therapeutic approach. However, certain deficiencies remain in the known oligonucleotides, and these deficiencies can limit the effectiveness of such

10 oligonucleotides as therapeutic agents. For example, Wickstrom (*J. Biochem. Biophys. Meth.* (1986) 13:97-102) teaches that oligonucleotide phosphodiester are susceptible to nuclease-mediated degradation, thereby limiting their

15 bioavailability *in vivo*. Agrawal et al. (*Proc. Natl. Acad. Sci. (USA)* (1990) 87:1401-1405) teaches that oligonucleotide phosphoramidates or methylphosphonates when hybridized to RNA do not activate RNase H, the activation of which can be

20 important to the function of antisense oligonucleotides. Thus, a need for methods of controlling gene expression exists which uses oligonucleotides with improved therapeutic characteristics.

25 Several reports have been published on the development of phosphorothioate-linked oligonucleotides as potential anti-AIDS therapeutic agents. Although extensive studies on

30 chemical and molecular mechanisms of oligonucleotides have demonstrated the potential value of this novel therapeutic strategy, little is known about the pharmacokinetics and metabolism of these compounds *in vivo*.

Several preliminary studies on this topic  
 have been published. Agrawal et al. (*Proc. Natl. Acad.  
 Sci. (USA)* (1991) 88:7595-7599) describes the  
 intravenously and intraperitoneally administration  
 to mice of a 20mer phosphorothioate linked-  
 oligonucleotide. In this study, approximately 30%  
 of the administered dose was excreted in the urine  
 over the first 24 hours with accumulation  
 preferentially in the liver and kidney. Plasma  
 half-lives ranged from about 1 hour ( $t_{1/2\alpha}$ ) and 40  
 hours ( $t_{1/2\beta}$ ), respectively. Similar results have  
 been reported in subsequent studies (Iversen  
 (1991) *Anti-Cancer Drug Design* 6:531-538; Iversen  
 (1994) *Antisense Res. Devel.* 4:43-52; and Sands (1994)  
*Mol. Pharm.* 45:932-943). However, stability  
 problems may exist when oligonucleotides are  
 administered intravenously and intraperitoneally.  
 More recently, Agrawal et al. reported that  
 oligonucleotide hybrids containing 2'-O-methyl  
 ribonucleotides  
 at both the 3'- and 5' ends and  
 deoxyribonucleotide phosphorothioates in the  
 interior portion were absorbed through the  
 gastrointestinal (GI) tract of rats (*Biochem.  
 Pharm.* (1995) 50:571-576).

Thus, there remains a need to develop more  
 effective therapeutic methods of down-regulating  
 the expression of genes which can be easily  
 manipulated to fit the animal and condition to be  
 treated, and the gene to be targeted. Preferably,  
 these methods should be simple, painless, and  
 precise in effecting the target gene.

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SUMMARY OF THE INVENTION

5 The present invention provides a method of  
down-regulating the expression of a gene in an  
animal which involves the administration of an  
oligonucleotide complementary to the gene via an  
oral route, thereby bypassing the complications  
10 which may be experienced during intravenous and  
other modes of *in vivo* administration.

It has been discovered that hybrid  
oligonucleotides with other than phosphodiester  
bonds and having at least one 2'-substituted  
15 ribonucleotide and chimeric oligonucleotides with  
at least two different types of internucleotide  
linkages are relatively stable *in vivo* following  
oral administration to an animal, and that these  
molecules are successfully absorbed from the  
20 gastrointestinal tract and distributed to various  
body tissues. This discovery has been exploited  
to develop the present invention, which is a  
method of down-regulating the expression of a gene  
in an animal.

25 This method is also a means of examining the  
function of various genes in an animal, including  
those essential to animal development. Presently,  
gene function can only be examined by the arduous  
30 task of making a "knock out" animal such as a  
mouse. This task is difficult, time-consuming and  
cannot be accomplished for genes essential to  
animal development since the "knock out" would

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produce a lethal phenotype. The present invention overcomes the shortcomings of this model.

5 In the method of the invention, a pharmaceutical formulation containing an oligonucleotide complementary to the targeted gene is orally administered in a pharmaceutically acceptable carrier to the animal harboring the gene. The oligonucleotide inhibits the expression  
10 of the gene, thereby down-regulating its expression.

15 For purposes of the invention, the term "animal" is meant to encompass humans as well as other mammals, as well as reptiles amphibians, and insects. The term "oral administration" refers to the provision of the formulation via the mouth through ingestion, or via some other part of the gastrointestinal system including the  
20 esophagus.

25 As used herein, the term "oligonucleotide" is meant to include polymers of two or more nucleotides or nucleotide analogs connected together via 5' to 3' internucleotide linkages which may include any linkages that are known in the antisense art, including non-phosphodiester linkages. Such molecules have a 3' terminus and a  
30 5' terminus.

30 The term ~~"non-phosphodiester-linkages"~~ as used herein refers to a synthetic covalent attachment between the 5' end of one nucleotide and the 3' end of another nucleotide in which the

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5' nucleotide phosphate has been replaced with any number of chemical groups. Preferable synthetic linkages include alkylphosphonates, phosphorothioates, phosphorodithioates, alkylphosphonothioates, phosphoramidates, phosphoramidites, phosphate esters, carbamates, carbonates, phosphate triesters, acetamidate, and carboxymethyl esters. In one preferred embodiment of the invention, the all of the nucleotides of the oligonucleotide comprises are linked via phosphorothioate and/or phosphorodithioate linkages.

In some embodiments of the invention, the oligonucleotides administered are modified with other than, or in addition to, non-phosphodiester-internucleotide linkages. As used herein, the term "modified oligonucleotide" encompasses oligonucleotides with modified nucleic acid(s), base(s), and/or sugar(s) other than those found in nature. For example, a 3', 5'-substituted oligonucleotide is an oligonucleotide having a sugar which, at both its 3' and 5' positions is attached to a chemical group other than a hydroxyl group (at its 3' position) and other than a phosphate group (at its 5' position).

A modified oligonucleotide may also be one with added substituents such as diamines, cholesteryl, or other lipophilic groups, or a capped species. In addition, unoxidized or partially oxidized oligonucleotides having a substitution in one nonbridging oxygen per nucleotide in the molecule are also considered to

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be modified oligonucleotides. Also considered as modified oligonucleotides are oligonucleotides having nuclease resistance-conferring bulky substituents at their 3' and/or 5' end(s) and/or various other structural modifications not found *in vivo* without human intervention are also considered herein as modified.

In one embodiment, the oligonucleotide being administered in the method of the invention has non-phosphodiester internucleotide linkages and includes at least one 2'-substituted ribonucleotide.

For purposes of the invention, the term "2'-substituted oligonucleotide" refers to an oligonucleotide having a sugar attached to a chemical group other than a hydroxyl group at its 2' position. The 2'-OH of the ribose molecule can be substituted with -O-lower alkyl containing 1-6 carbon atoms, aryl or substituted aryl or allyl having 2-6 carbon atoms, e.g., 2'-O-allyl, 2'-O-aryl, 2'-O-alkyl (such as a 2'-O-methyl), 2'-halo, or 2'-amino, but not with 2'-H, wherein allyl, aryl, or alkyl groups may be unsubstituted or substituted, e.g., with halo, hydroxy, trifluoromethyl, cyano, nitro, acyl, acyloxy, alkoxy, carboxyl, carbalkoxyl or amino groups.

In one preferred embodiment of the invention, the oligonucleotide administered includes at least one 2'-substituted ribonucleotide at its 3' terminus. In some embodiments, all but four or five nucleotides at its 5' terminus are 2'-

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substituted ribonucleotides, and in some  
embodiments, these four or five unsubstituted 5'  
nucleotides are deoxyribonucleotides. In other  
embodiments, the oligonucleotide has at least one  
2'-substituted ribonucleotide at both its 3' and  
5' termini, and in yet other embodiments, the  
oligonucleotide is composed of 2'-substituted  
ribonucleotides in all positions with the  
exception of at least four or five contiguous  
deoxyribonucleotide nucleotides in any interior  
position. Another aspect of the invention  
includes the administration of an oligonucleotide  
composed of nucleotides that are all 2'-  
substituted ribonucleotides. Particular  
embodiments include oligonucleotides having a 2'-  
O-alkyl-ribonucleotide such as a 2'-O methyl.  
Other embodiments include the administration of  
chimeric oligonucleotides. In one preferred  
embodiment, the chimeric oligonucleotide has at  
least one alkylphosphonate internucleotide linkage  
at both its 3' and 5' ends and having  
phosphorothioate internucleotide linkages.

In another embodiment of the invention, the  
oligonucleotide administered has at least one  
deoxyribonucleotide, and in a preferred  
embodiment, the oligonucleotide has at least four  
or five contiguous deoxyribonucleotides capable of  
activating RNase H.

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The oligonucleotide administered is  
complementary to a gene of a virus, pathogenic  
organism, or a cellular gene in some embodiments  
of the invention. In some embodiments, the

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oligonucleotide is complementary to a gene of a virus involved in AIDS, oral or genital herpes, papilloma warts, influenza, foot and mouth disease, yellow fever, chicken pox, shingles, adult T-cell leukemia, Burkitt's lymphoma, nasopharyngeal carcinoma, or hepatitis. In one particular embodiment, the oligonucleotide is complementary to an HIV gene and includes about 15 to 26 nucleotides linked by phosphorothioate internucleotide linkages, at least one of the nucleotides at the 3' terminus being a 2'-substituted ribonucleotide, and at least four contiguous deoxyribonucleotides.

15 <sup>SUB</sup> ~~Alc~~ In another embodiment, the oligonucleotide is complementary to a gene encoding a protein in associated with Alzheimer's disease.

20 In yet other embodiments, the oligonucleotide is complementary to a gene encoding a protein expressed in a parasite that causes a parasitic disease such as amebiasis, Chagas' disease, toxoplasmosis, pneumocytosis, giardiasis, cryptosporidiosis, trichomoniasis, malaria, 25 ascariasis, filariasis, trichinosis, or schistosomiasis infections.

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The foregoing and other objects of the present invention, the various features thereof, as well as the invention itself may be more fully understood from the following description, when read together with the accompanying drawings in which:

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FIG. 3B is a representation of an autoradiogram of radiolabelled oligonucleotide in the stomach, small intestine, and large intestine of mice at different times following oral administration of chimeric oligonucleotide;

FIG. 4A is an HPLC chromatograph of radiolabelled PS oligonucleotide standard;

FIG. 4B is an HPLC chromatograph of oligonucleotides extracted from plasma samples taken 12 hours after the administration of radiolabelled PS oligonucleotide;

FIG. 5A is an HPLC chromatograph of radiolabelled PS oligonucleotide standard;

FIG. 5B is an HPLC chromatograph of oligonucleotides extracted from rat liver 6 hours after the administration of radiolabelled PS oligonucleotide;

FIG. 5C is an HPLC chromatograph of oligonucleotides extracted from rat liver 24 hours after the administration of radiolabelled PS oligonucleotide;

FIG. 6 is a graphic representation demonstrating the time course of urinary excretion of radioactivity in rats following the oral administration of radiolabelled PS oligonucleotide;

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FIG. 7A is an HPLC chromatogram of radiolabelled PS oligonucleotide standard;

5        FIG. 7B is an HPLC chromatogram of oligonucleotides extracted from rat urine 6 hours after the administration of radiolabelled PS oligonucleotide;

10       FIG. 7C is an HPLC chromatogram of oligonucleotides extracted from rat urine 12 hours after the administration of radiolabelled PS oligonucleotide;

15       FIG. 8 is a graphic representation showing the course of radioactivity in the gastrointestinal tract and feces in rats following the oral administration of radiolabelled PS oligonucleotide;

20       FIG. 9 is an HPLC chromatogram of oligonucleotides extracted from rat stomach 1 hour, 3 hours, and 6 hours after the administration of radiolabelled PS oligonucleotide;

25       FIG. 10 is an HPLC chromatogram of oligonucleotides extracted from rat large intestine 3 hours, 6 hours, and 12 hours after the administration of radiolabelled PS  
30       oligonucleotide;

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FIG. 11A is a representation of an autoradiogram of radiolabelled oligonucleotide in the plasma, liver, kidney, spleen, heart, and lung of mice 6 hours following oral administration of hybrid oligonucleotide;

FIG. 11B is a representation of an autoradiogram of radiolabelled oligonucleotide in the plasma, liver, kidney, spleen, heart, and lung of mice 6 hours following oral administration of chimeric oligonucleotide; and

FIG. 12 is a graphic representation of the distribution of radioactivity in GI + feces, plasma, tissue, and urine at various times following oral administration of PS oligonucleotide (30 mg/kg rat), hybrid oligonucleotide (10 mg/kg mouse), and chimeric oligonucleotide (10 mg/kg mouse).

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DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

5 The patent and scientific literature referred to herein establishes the knowledge that is available to those with skill in the art. The issued U.S. patent, allowed patent applications, and articles cited herein are hereby incorporated by reference.

10 This invention provides a method of down-regulating the expression of a gene in an animal by the oral administration of an oligonucleotide whose nucleotide sequence is complementary to the targeted gene.

15 It is known that an oligonucleotide, called an "antisense oligonucleotide," can bind to a target single-stranded nucleic acid molecule according to the Watson-Crick or the Hoogsteen  
20 rule of base pairing, and in doing so, disrupt the function of the target by one of several mechanisms: by preventing the binding of factors required for normal transcription, splicing, or translation; by triggering the enzymatic  
25 destruction of mRNA by RNase H if a contiguous region of deoxyribonucleotides exists in the oligonucleotide, and/or by destroying the target via reactive groups attached directly to the antisense oligonucleotide.

30 Thus, because of the properties described above, such oligonucleotides are useful therapeutically by their ability to control or down-regulate the expression of a particular gene

in an animal, according to the method of the present invention.

5 The oligonucleotides useful in the method of the invention are at least 6 nucleotides in length, but are preferably 6 to 50, more preferably 11 to 35, most preferably 15 to 30, and commonly 15 to 25 nucleotides in length. They are composed of deoxyribonucleotides, ribonucleotides, 10 or a combination of both, with the 5' end of one nucleotide and the 3' end of another nucleotide being covalently linked by non-phosphodiester internucleotide linkages. Such linkages include alkylphosphonates, phosphorothioates, 15 phosphorodithioates, alkylphosphonothioates, alkylphosphonates, phosphoramidates, phosphate esters, carbamates, acetamidate, carboxymethyl esters, carbonates, and phosphate triesters. Oligonucleotides with these linkages can be prepared according to known methods such as 20 phosphoramidate or H-phosphonate chemistry which can be carried out manually or by an automated synthesizer as described by Brown (*A Brief History of Oligonucleotide Synthesis. Protocols for Oligonucleotides and Analogs, Methods in Molecular Biology* (1994) 20:1-8). (See also, 25 e.g., Sonveaux "Protecting Groups in Oligonucleotides Synthesis" in Agrawal (1994) *Methods in Molecular Biology* 26:1-72; Uhlmann et al. (1990) *Chem. Rev.* 90:543-583).

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The oligonucleotides of the composition may also be modified in a number of ways without compromising their ability to hybridize to the

target nucleic acid. Such modifications include, for example, those which are internal or at the end(s) of the oligonucleotide molecule and include additions to the molecule of the internucleoside phosphate linkages, such as cholesteryl or diamine compounds with varying numbers of carbon residues between the amino groups and terminal ribose, deoxyribose and phosphate modifications which cleave, or crosslink to the opposite chains or to associated enzymes or other proteins which bind to the viral genome. Examples of such modified oligonucleotides include oligonucleotides with a modified base and/or sugar such as arabinose instead of ribose, or a 3', 5'-substituted oligonucleotide having a sugar which, at both its 3' and 5' positions is attached to a chemical group other than a hydroxyl group (at its 3' position) and other than a phosphate group (at its 5' position). Other modified oligonucleotides are capped with a nuclease resistance-conferring bulky substituent at their 3' and/or 5' end(s), or have a substitution in one nonbridging oxygen per nucleotide. Such modifications can be at some or all of the internucleoside linkages, as well as at either or both ends of the oligonucleotide and/or in the interior of the molecule. For the preparation of such modified oligonucleotides, see, e.g., Agrawal (1994) *Methods in Molecular Biology* 26; Uhlmann et al. (1990) *Chem. Rev.* 90:543-583).

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Oligonucleotides which are self-stabilized are also considered to be modified oligonucleotides useful in the methods of the invention (Tang et al. (1993) *Nucleic Acids Res.*

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20:2729-2735). These oligonucleotides comprise two regions: a target hybridizing region; and a self-complementary region having an oligonucleotide sequence complementary to a nucleic acid sequence that is within the self-stabilized oligonucleotide.

The preparation of these unmodified and modified oligonucleotides is well known in the art (reviewed in Agrawal et al. (1992) *Trends Biotechnol.* 10:152-158) (see, e.g., Uhlmann et al. (1990) *Chem. Rev.* 90:543-584; and (1987) *Tetrahedron. Lett.* 28:(31):3539-3542); Agrawal (1994) *Methods in Molecular Biology* 20:63-80); and Zhang et al. (1996) *J. Pharmacol. Expt. Thera.* 278:1-5).

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The oligonucleotides administered to the animal may be hybrid oligonucleotides in that they contain both deoxyribonucleotides and at least one 2' substituted ribonucleotide. For purposes of the invention, the term "2'-substituted" means substitution at the 2' position of the ribose with, e.g., a -O-lower alkyl containing 1-6 carbon atoms, aryl or substituted aryl or allyl having 2-6 carbon atoms e.g., 2'-O-allyl, 2'-O-aryl, 2'-O-alkyl, 2'-halo, or 2'-amino, but not with 2'-H, wherein allyl, aryl, or alkyl groups may be unsubstituted or substituted, e.g., with halo, hydroxy, trifluoromethyl, cyano, nitro, acyl, acyloxy, alkoxy, carboxyl, carbalkoxyl or amino groups. Useful substituted ribonucleotides are 2'-O-alkyls such as 2'-O-methyl.

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5 The hybrid DNA/RNA oligonucleotides useful in the method of the invention resist nucleolytic degradation, form stable duplexes with RNA or DNA, and preferably activate RNase H when hybridized with RNA. They may additionally include at least one unsubstituted ribonucleotide. For example, an oligonucleotide useful in the method of the invention may contain all deoxyribonucleotides with the exception of one 2' substituted ribonucleotide at the 3' terminus of the oligonucleotide. Alternatively, the oligonucleotide may have at least one substituted ribonucleotide at both its 3' and 5' termini.

15 One preferred class of oligonucleotides useful in the method of the invention contains four or more deoxyribonucleotides in a contiguous block, so as to provide an activating segment for RNase H. In certain cases, more than one such activating segment will be present at any location within the oligonucleotide. There may be a majority of deoxyribonucleotides in oligonucleotides according to the invention. In fact, such oligonucleotides may have as many as all but one nucleotide being deoxyribonucleotides. Thus, a preferred oligonucleotide having from about 2 to about 50 nucleotides or most preferably from about 12 to about 25 nucleotides, the number of deoxyribonucleotides present ranges from 1 to about 24. Other useful oligonucleotides may consist only of 2'-substituted ribonucleotides.

TABLE 1 lists some representative species of oligonucleotides which are useful in the method of

the invention. 2'-substituted nucleotides are  
underscored.

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TABLE 1

OLIGO NO.	OLIGONUCLEOTIDE	SEQ ID NO.:
5	1 CTCTCGCACCCATCTCTCCTTCU	1
	2 CTCTCGCACCCATCTCTCCTUCU	2
	3 CTCTCGCACCCATCTCTCCUUCU	3
	4 CTCTCGCACCCATCTCUCUCCUUCU	4
	5 CTCTCGCACCCAUCUCUCUCCUUCU	5
10	6 CTCTCGCACCCAUCUCUCUCCUUCU	6
	7 CTCTCGCACCCAUCUCUCUCCUUCU	7
	8 CUCUCGCACCCAUCUCUCUCCUUCU	8
	9 CTCTCGCACCCATCTCTCCTTCU	1
	10 CUCTCGCACCCATCTCTCCTTCU	9
15	11 CUCUCGCACCCATCTCTCCTTCU	10
	12 CUCUCGCACCCATCTCUCUCCUUCU	11
	13 CUCUCGCACCCAUCUCUCUCCUUCU	12
	14 CUCUCGCACCCATCTCTCUCUCCUUCU	13
	15 CTCTCGCACCCAUCUCUCUCCUUCU	5
20	16 CUCUCGCACCCAUCTCTCTCCUUCU	14
	17 CUCUCGCACCCATCTCTCTCCUUCU	15
	18 CUCTCGCACCCAUCUCUCUCCUUCU	16
	19 CUCTCGCACCCATCTCTCUCUCCUUCU	17
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The oligonucleotides administered to the animal may be chimeric in that they contain more than one type of internucleotide linkage. Such chimeric oligonucleotides are described in U.S. Patent Nos. 5,149,797 and 5,366,878. For example, chimeric oligonucleotides useful in the method of the invention may include phosphorothioate and alkylphosphonate internucleotide linkages. One preferred alkylphosphonate linkage is a methylphosphonate linkage.

Table 2 lists some representative specifics of chimeric oligonucleotides which are useful in the method of the invention. The alkylphosphonate internucleotide linkages are indicated by ":"; the phosphorothioate linkages are indicated by "-".

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TABLE 2

NO:	OLIGONUCLEOTIDE (5'→3')	SEQ ID NO:
20	C:T:C:T-C-G-C-A-C-C-C-A-T-C-T-C-T-C-T-C-C-T:T:C:T	18
21	C:T:C:T-C-G-C-A-C-C-C-A-T-C-T-C-T-C-T-C-C-T:T:C:T	18
22	C:T:C:T-C-G-C-A-C-C-C-A-T-C-T-C-T-C-T-C-C-T:T:C:T	18
23	C:T:C-T-C-G-C-A-C-C-C-A-T-C-T-C-T-C-T-C-C-T:T:C:T	18
24	C:T:C-T-C-G-C-A-C-C-C-A-T-C-T-C-T-C-T-C-C-T:T:C:T	18
25	C:T:C-T-C-G-C-A-C-C-C-A-T-C-T-C-T-C-T-C-C-T:T:C:T	18
26	C:T:C-T-C-G-C-A-C-C-C-A-T-C-T-C-T-C-T-C-C-T:T:C:T	18
27	C:T:C-T-C-G-C-A-C-C-C-A-T-C-T-C-T-C-T-C-C-T:T:C:T	18
28	C:T:C-T-C-G-C-A-C-C-C-A-T-C-T-C-T-C-T-C-C-T:T:C:T	18
29	C:T:C-T-C-G-C-A-C-C-C-A-T-C-T-C-T-C-T-C-C-T:T:C:T	18
30	C:T:C-T-C-G-C-A-C-C-C-A-T-C-T-C-T-C-T-C-C-T:T:C:T	18
31	C:T:C-T-C-G-C-A-C-C-C-A-T-C-T-C-T-C-T-C-C-T:T:C:T	18
32	C:T:C-T-C-G-C-A-C-C-C-A-T-C-T-C-T-C-T-C-C-T:T:C:T	18

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TABLE 2 (CON'T)

NO:	OLIGONUCLEOTIDE (5'-3')	SEQ ID NO:
33	C:T:C:T-C-G-C-A-C-C-C-A-T-C-T-C-T-C-T-C-C-T	19
34	C:T:C:T-C-G-C-A-C-C-C-A-T-C-T-C-T-C-T-C-C-T	19
35	C:T:C:T-C-G-C-A-C-C-C-A-T-C-T-C-T-C-T-C-C-T	19
36	C:T:C-T-C-G-C-A-C-C-C-A-T-C-T-C-T-C-T-C-C-T	19
37	C:T:C-T-C-G-C-A-C-C-C-A-T-C-T-C-T-C-T-C-C-T	19
38	C:T:C:T-C-G-C-A-C-C-C-A-T-C-T-C-T-C-T-C-C-T	19
39	C:T:C:T-C-G-C-A-C-C-C-A-T-C-T-C-T-C-T-C-C-T	19
40	C:T:C-T-C-G-C-A-C-C-C-A-T-C-T-C-T-C-T-C-C-T	19
41	C:T:C:T-C-G-C-A-C-C-C-A-T-C-T-C-T-C-T-C-C-T	19
42	C:T-C-T-C-G-C-A-C-C-C-A-T-C-T-C-T-C-T-C-C-T	19
43	C:T-C-T-C-G-C-A-C-C-C-A-T-C-T-C-T-C-T-C-C-T	19
44	C:T:C:T-C-G-C-A-C-C-C-A-T-C-T-C-T-C-T-C-C-T	19
45	C:T:C:T-C-G-C-A-C-C-C-A-T-C-T-C-T-C-T-C-C-T	19

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The oligonucleotides according to the invention are effective in inhibiting the expression of various genes in viruses, pathogenic organisms, or in inhibiting the expression of cellular genes. The ability to inhibit such agents is clearly important to the treatment of a variety of disease states. Thus, oligonucleotides according to the method of the invention have a nucleotide sequence which is complementary to a nucleic acid sequence that is from a virus, a pathogenic organism or a cellular gene. Preferably such oligonucleotides are from about 6 to about 50 nucleotides in length.

For purposes of the invention, the term "oligonucleotide sequence that is complementary to a nucleic acid sequence" is intended to mean an oligonucleotide sequence that binds to the target nucleic acid sequence under physiological conditions, e.g., by Watson-Crick base pairing (interaction between oligonucleotide and single-stranded nucleic acid) or by Hoogsteen base pairing (interaction between oligonucleotide and double-stranded nucleic acid) or by any other means including in the case of a oligonucleotide binding to RNA, pseudoknot formation. Such binding (by Watson Crick base pairing) under physiological conditions is measured as a practical matter by observing interference with the function of the nucleic acid sequence.

The nucleic acid sequence to which an oligonucleotide according to the invention is complementary will vary, depending upon the gene

to be down-regulated. In some cases, the target gene or nucleic acid sequence will be a virus nucleic acid sequence. The use of antisense oligonucleotides to inhibit various viruses is well known (reviewed in Agrawal (1992) *Trends in Biotech.* 10:152-158). Viral nucleic acid sequences that are complementary to effective antisense oligonucleotides have been described for many viruses, including human immunodeficiency virus type 1 (HIV-1) (U.S. Patent No. 4,806,463), herpes simplex virus (U.S. Patent No. 4,689,320), influenza virus (U.S. Patent No. 5,194,428), and human papilloma virus (Storey et al. (1991) *Nucleic Acids Res.* 19:4109-4114 ). Sequences complementary to any of these nucleic acid sequences can be used for oligonucleotides according to the invention, as can be oligonucleotide sequences complementary to nucleic acid sequences from any other virus. Additional viruses that have known nucleic acid sequences against which antisense oligonucleotides can be prepared include, but are not limited to, foot and mouth disease virus (see, Robertson et al. (1985) *J. Virol.* 54:651; Harris et al. (1980) *Virol.* 36:659), yellow fever virus (see Rice et al. (1985) *Science* 229:726), varicella-zoster virus (see, Davison and Scott (1986) *J. Gen. Virol.* 67:2279), Epstein-Barr virus, cytomegalovirus, respiratory syncytial virus (RSV), and cucumber mosaic virus (see Richards et al. (1978) *Virol.* 89:395).

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For example, an oligonucleotide has been designed which is complementary to a portion of the HIV-1 gene, and as such, has significant anti-HIV effects (Agrawal (1992) *Antisense Res. Development* 2:261-266). The target of this oligonucleotide has been found to be conserved among various HIV-1 isolates. It is 56% G + C rich, water soluble, and relatively stable under physiological conditions. This oligonucleotide binds to a complementary RNA target under physiological conditions, with the T of the duplex approximately being 56°C. The antiviral activity of this oligonucleotide has been tested in several models, including acutely and chronically infected CEM cells, long-term cultures mimicking *in vivo* conditions, human peripheral blood lymphocytes and macrophages, and isolates from HIV-1 infected patients (Liszewicz et al. (*Proc. Natl. Acad. Sci. (USA)* (1992) 89:11209-11213); Liszewicz et al. (*Proc. Natl. Acad. Sci. (USA)* (1993) 90:3860-3864); Liszewicz et al. (*Proc. Natl. Acad. Sci. (USA)* (1994) 91:7942-7946); Agrawal et al. (*J. Ther. Biotech*) in press).

The oligonucleotides according to the invention alternatively can have an oligonucleotide sequence complementary to a nucleic acid sequence of a pathogenic organism. The nucleic acid sequences of many pathogenic organisms have been described, including the malaria organism, *Plasmodium falciparum*, and many pathogenic bacteria. Oligonucleotide sequences complementary to nucleic acid sequences from any such pathogenic organism can be used in

oligonucleotides according to the invention. Nonlimiting examples of pathogenic eucaryotes having known nucleic acid sequences against which antisense oligonucleotides can be prepared include

5 *Trypanosom abrucei gambiense* and *Leishmania* (See Campbell et al., *Nature* 311:350 (1984)), and *Fasciola hepatica* (See Zurita et al., *Proc. Natl. Acad. Sci. USA* 84:2340 (1987)).

10 Antifungal oligonucleotides can be prepared using a target hybridizing region having an oligonucleotide sequence that is complementary to a nucleic acid sequence from, e.g., the chitin synthetase gene, and antibacterial

15 oligonucleotides can be prepared using, e.g., the alanine racemase gene. Among fungal diseases that may be treatable by the method of treatment according to the invention are candidiasis, histoplasmosis, cryptococcocis, blastomycosis,

20 aspergillosis, sporotrichosis, chromomycosis, dermatophytosis, and coccidioidomycosis. The method might also be used to treat rickettsial diseases (e.g., typhus, Rocky Mountain spotted fever), as well as sexually transmitted diseases

25 caused by *Chlamydia trachomatis* or *Lymphogranuloma venereum*. A variety of parasitic diseases may be treated by the method according to the invention, including amebiasis, Chagas' disease, toxoplasmosis, pneumocystosis, giardiasis,

30 cryptosporidiosis, trichomoniasis, and *Pneumocystis carini* pneumonia; also worm (helminthic) diseases such as ascariasis, filariasis, trichinosis, schistosomiasis and nematode or cestode infections. Malaria may be

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treated by the method of treatment of the invention regardless of whether it is caused by *P. falciparum*, *P. vivax*, *P. ovale*, or *P. malariae*.

5           The infectious diseases identified above may all be treated by the method of treatment according to the invention because the infectious agents for these diseases are known and thus oligonucleotides according to the invention can be prepared, having oligonucleotide sequence that is complementary to a nucleic acid sequence that is an essential nucleic acid sequence for the propagation of the infectious agent, such as an essential gene.

15           Other disease states or conditions that may be treatable by the method according to the invention are those which result from an abnormal expression or product of a cellular gene. These conditions may be treated by administration of oligonucleotides according to the invention, and have been discussed earlier in this disclosure.

25           Other oligonucleotides according to the invention can have a nucleotide sequence complementary to a cellular gene or gene transcript, the abnormal expression or product of which results in a disease state. The nucleic acid sequences of several such cellular genes have been described, including prion protein (Stahl et al. (1991) *FASEB J.* 5:2799-2807), the amyloid-like protein associated with Alzheimer's disease (U.S. Patent No. 5,015,570), and various well-known oncogenes and proto-oncogenes, such as *c-myc*, *c-*

myc, c-abl, and n-ras. In addition,  
oligonucleotides that inhibit the synthesis of  
structural proteins or enzymes involved largely or  
exclusively in spermatogenesis, sperm motility,  
5 the binding of the sperm to the egg or any other  
step affecting sperm viability may be used as  
contraceptives. Similarly, contraceptives for  
women may be oligonucleotides that inhibit  
proteins or enzymes involved in ovulation,  
10 fertilization, implantation or in the biosynthesis  
of hormones involved in those processes.

Hypertension may be controlled by  
oligonucleotides that down-regulate the synthesis  
15 of angiotensin converting enzyme or related  
enzymes in the renin/angiotensin system. Platelet  
aggregation may be controlled by suppression of  
the synthesis of enzymes necessary for the  
synthesis of thromboxane A2 for use in myocardial  
20 and cerebral circulatory disorders, infarcts,  
arteriosclerosis, embolism and thrombosis.  
Deposition of cholesterol in arterial wall may be  
inhibited by suppression of the synthesis of fatty  
acid co-enzyme A: cholesterol acyl transferase in  
25 arteriosclerosis. Inhibition of the synthesis of  
cholinephosphotransferase may be useful in  
hypolipidemia.

There are numerous neural disorders in which  
30 hybridization arrest may be used to reduce or  
eliminate adverse effects of the disorder. For  
example, suppression of the synthesis of monoamine  
oxidase may be used in Parkinson's disease.  
Suppression of catechol o-methyl transferase may

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be used to treat depression; and suppression of indole N-methyl transferase may be used in treating schizophrenia.

5           Suppression of selected enzymes in the arachidonic acid cascade which leads to prostaglandins and leukotrienes may be useful in the control of platelet aggregation, allergy, inflammation, pain and asthma.

10           Suppression of the protein expressed by the multidrug resistance (*mdr-1*) gene, which can be responsible for development of resistance of tumors to a variety of anti-cancer drugs and is a major impediment in chemotherapy may prove to be beneficial in the treatment of cancer.

15           Oligonucleotide sequences complementary to nucleic acid sequences from any of these genes can be used for oligonucleotides according to the invention, as can be oligonucleotide sequences complementary

20           to any other cellular gene transcript, the abnormal expression or product of which results in a disease state.

25           The oligonucleotides described herein are administered orally or enterally to the animal subject in the form of therapeutic pharmaceutical formulations that are effective for treating virus infection, infections by pathogenic organisms, or

30           disease resulting from abnormal gene expression or from the expression of an abnormal gene product. In some aspects of the method according to the invention, the oligonucleotides are administered

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in conjunction with other therapeutic agents,  
e.g., AZT in the case of AIDS.

5       The therapeutic pharmaceutical formulation  
containing the oligonucleotide includes a  
physiologically acceptable carrier, such as an  
inert diluent or an assimilable edible carrier  
with which the peptide is administered. Suitable  
10       formulations that include pharmaceutically  
acceptable excipients for introducing compounds to  
the bloodstream by other than injection routes can  
be found in *Remington's Pharmaceutical Sciences* (18th ed.)  
(Genarro, ed. (1990) Mack Publishing Co., Easton,  
15       PA). The oligonucleotide and other ingredients  
may be enclosed in a hard or soft shell gelatin  
capsule, compressed into tablets, or incorporated  
directly into the individual's diet. The  
oligonucleotide may be incorporated with  
20       excipients and used in the form of ingestible  
tablets, buccal tablets, troches, capsules,  
elixirs, suspensions, syrups, wafers, and the  
like. When the oligonucleotide is administered  
orally, it may be mixed with other food forms and  
pharmaceutically acceptable flavor enhancers.  
25       When the oligonucleotide is administered  
enterally, they may be introduced in a solid,  
semi-solid, suspension, or emulsion form and may  
be compounded with any number of well-known,  
pharmaceutically acceptable additives. Sustained  
30       release oral delivery systems and/or enteric  
coatings for orally administered dosage forms are  
also contemplated such as those described in U.S.  
Patent Nos. 4,704,295, 4,556,552, 4,309,404, and  
4,309,406.

The amount of oligonucleotide in such therapeutically useful compositions is such that a suitable dosage will be obtained. Preferred compositions or preparations according to the present invention are prepared so that an oral dosage unit contains between from about 50 micrograms to about 200 mg per kg body weight of the animal, with 10 mg to 100 mg per kg being most preferable.

It will be appreciated that the unit content of active ingredient or ingredients contained in an individual dose of each dosage form need not in itself constitute an effective amount since the necessary effective amount can be reached by administration of a plurality of dosage units (such as capsules or tablets or combinations thereof).

In order to determine if the oligonucleotide administered according to the method of the invention is absorbed into body tissues, and if so, in which tissues absorption occurs, the following study was performed. Samples of various body tissues from treated rats were analyzed for radioactivity at increasing hours after oral administration of a radioactively labelled phosphorothioate oligonucleotide. FIG. 1 illustrates the plasma, liver, and kidney concentration-time course of an oligonucleotide equivalents after oral administration of the radiolabelled oligonucleotide. These results demonstrate that the drug is absorbed through

gastrointestinal tract and accumulated in the kidney and the liver.

As illustrated in FIGS. 2A and 2B, both unmodified and hybrid oligonucleotides were shown to be stable in the stomach up to 6 hr following oral administration. The unmodified oligonucleotide underwent extensive degradation in small and large intestine, the majority of the radioactivity being associated with the different length of truncated oligonucleotide (FIG. 2A). In contrast, the hybrid oligonucleotide was more stable compared to the unmodified oligonucleotide, the majority of the radioactivity in small intestine being associated with the intact oligonucleotide (FIG. 2B). Increased degradation of the hybrid oligonucleotide was observed in the large intestine (FIG. 2B).

<sup>35</sup>S-labelled modified oligonucleotides were also orally administered to mice at a single dose. For the hybrid oligonucleotide, similar profiles of gel electrophoresis of radioactivity in the gastrointestinal tract were observed with mice compared to rats (FIG. 3A). For the chimeric oligonucleotide, gel electrophoresis of radioactivity in the gastrointestinal tract revealed that this compound was stable in stomach and small intestine, with significant degradation in large intestine (FIG. 3B).

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The chemical form of radioactivity in rat plasma was further evaluated by HPLC as shown is FIG. 4A and 4B, demonstrating the presence of both

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Radioactivity in rat brain, thymus, heart, lung, liver, kidney, adrenals, stomach, small intestine, large intestine, skeletal muscle, testes, thyroid, epidermis, whole eye, and bone marrow was detectable 48 hours after oral administration of the radiolabelled oligonucleotide. For unmodified oligonucleotide, minimal intact form was detectable in rat tissue samples. However, as shown in FIG. 11A for the hybrid oligonucleotide and in FIG. 11B for the chimeric oligonucleotide, intact oligonucleotides were detected in plasma and tissue samples of the liver, kidney, spleen, heart, and lung.

Further evidence to support the absorption of the oligonucleotide comes from urine sample analysis after radioactively labelled oligonucleotide was orally administered. FIG. 6 shows the cumulative excretion of the radioactively labelled oligonucleotide into the urine over 48 hr following the administration of radiolabelled phosphorothioate oligonucleotide. That the oligonucleotide continues to be excreted in the urine over time implies that other tissues had absorbed it, and that the body was capable of absorption for an extended period of time. FIGS. 7B and 7C demonstrate that although the majority of radioactivity in urine was present as degradative products, intact oligonucleotide was

also detected, demonstrating that this oligonucleotide is absorbed intact.

5 To determine the level of bioavailability of  
oligonucleotides following oral administration,  
the level of the oligonucleotide in the  
gastrointestinal tract (stomach and intestine) and  
feces was measured. FIG. 8 shows that  
10 approximately 80% of administered oligonucleotide  
remained or was excreted in feces, indicating that  
20% of administered oligonucleotide was absorbed.  
This oligonucleotide was stable in stomach; no  
significant degradative products in stomach  
15 contents were detected six hours after oral  
administration (FIG. 9). The majority of  
administered oligonucleotide in the contents of  
the large intestine were also present as the  
intact compound (FIG. 10).

20 In another study, the oral bioavailability of  
unmodified, hybrid, and chimeric oligonucleotide  
administered to rat and mouse were compared, based  
on the quantitation of radioactivity in the  
gastrointestinal tract, feces, plasma, urine and  
25 remaining tissues at various times. Total  
recovery of radioactivity in the study was  $92 \pm 6\%$ .  
The total absorption of unmodified  
oligonucleotide was shown to be  $17.3 \pm 5.5\%$  over 6  
hr and  $35.5 \pm 6.0\%$  over 12 hr following oral  
30 administration of the radiolabelled unmodified  
oligonucleotide to rats at a dose of 30 mg/kg.  
Minimal intact unmodified oligonucleotide was also  
detected in tissues outside enterohepatic system.

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5 The total absorption of hybrid  
oligonucleotide was determined to be  $10.2 \pm 2.5\%$   
over 6 hr and  $25.9 \pm 4.7\%$  over 12 hr following  
oral administration of the radiolabelled hybrid  
oligonucleotide in rats. Although the total  
absorption rates were slightly lower than that of  
the PS oligonucleotide, the hybrid  
oligonucleotide-derived radioactivity was stable  
in various tissues. The total absorption of the  
10 chimeric oligonucleotide was determined to be  $23.6$   
 $\pm 2.8\%$  over 6 hr and  $39.3 \pm 2.4\%$  over 12 hr  
following oral administration of the radiolabelled  
oligonucleotide. The comparison of oral  
availability of the three types of  
15 oligonucleotides is shown in FIG. 12, expressed  
as the percentages of administered doses in the  
gastrointestinal tract plus feces, in plasma, in  
tissues, and in urine.

20 Oral absorption of oligonucleotides in  
fasting animals was also determined with PS-  
oligonucleotide and hybrid oligonucleotide.  
Decreased absorption rates were found, indicating  
that the retention time of the oligonucleotides in  
the gastrointestinal tract in the fasting animals  
25 may be lower than in non-fasting animals.

30 These studies indicate that hybrid and  
chimeric oligonucleotides have enhanced  
bioavailability, which is associated with their  
stability in the gastrointestinal tract and other  
tissues.

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Thus, using the method of the invention, successful absorption of oligonucleotides was accomplished through the gastrointestinal tract and distributed throughout the body. Intact oligonucleotides were detected in plasma and various tissues and excreted into the urine. These results demonstrate that oral administration is a potential means for delivery of oligonucleotides as therapeutic agents.

The following examples illustrate the preferred modes of making and practicing the present invention, but are not meant to limit the scope of the invention since alternative methods may be utilized to obtain similar results.

#### EXAMPLES

##### 1. Synthesis and Analysis of Oligonucleotides

Sub A" 25  
An unmodified HIV-specific 25mer oligonucleotide and hybrid 25mer phosphorothioate-linked oligonucleotide having SEQ ID NO:10 and containing 2'-O-methyl ribonucleotide 3' and 5' sequences and a deoxyribonucleotide interior, as well as two hybrid 18mer phosphorothioate-linked oligonucleotides having SEQ ID NOS:20 and 21, and containing 2'-O-methyl ribonucleotide 3' and 5' sequences and a deoxyribonucleotide interior, were synthesized, purified, and analyzed as follows.

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Unmodified phosphorothioate deoxynucleosides were synthesized on CPG on a 5-6  $\mu$ mole scale on an automated synthesizer (model 8700, Millipore, Bedford, MA) using the H-phosphonate approach described in U.S. Patent No. 5,149,798.

Deoxynucleoside H-phosphonates were obtained from Millipore (Bedford, MA). 2'-O-methyl ribonucleotide H-phosphonates or phosphorothioates were synthesized by standard procedures (see, e.g., "Protocols for Oligonucleotides and Analogs" in *Meth. Mol. Biol.* (1993) volume 20) or commercially obtained (e.g., from Glenn Research, Sterling, VA and Clontech, Palo Alto, CA). Segments of oligonucleotides containing 2'-O-methyl nucleoside(s) were assembled by using 2'-O-methyl ribonucleoside H-phosphonates or phosphorothioates for the desired cycles. Similarly, segments of oligonucleotides containing deoxyribonucleosides were assembled by using deoxynucleoside H-phosphonates for the desired cycles. After assembly, CPG bound oligonucleotide H-phosphonate was oxidized with sulfur to generate the phosphorothioate linkage. Oligonucleotides were then deprotected in concentrated  $\text{NH}_4\text{OH}$  at 40°C for 48 hours.

Crude oligonucleotide (about 500  $A_{260}$  units) was analyzed on reverse low pressure chromatography on a  $C_{18}$  reversed phase medium. The DMT group was removed by treatment with 80% aqueous acetic acid, then the oligonucleotides were dialyzed against distilled water and lyophilized.

Chimeric oligonucleotide was prepared as described in Zhang et al. (*J. Pharmacol. Exptal. Thera.* (1996) 278:(in press)). This chimeric oligonucleotide had 3 methylphosphate internucleotide linkages at its 5' end, 4 methylphosphonate internucleotide linkages at its 3' end, and phosphorothioate internucleotide linkages elsewhere in the molecule were prepared and purified as follows. The first four couplings were carried out by using nucleoside methylphosphoramidite, followed by oxidation with a standard iodine reagent. The next seven couplings were carried out by using nucleoside  $\beta$ -cyanoethylphosphoramidite, followed by oxidation with 3H-1,2-benzodithiole-3-one-1,1,-dioxide. The eighth coupling was carried out by using nucleoside  $\beta$ -cyanoethylphosphoramidite. After several washes with acetonitrile, the column was removed from the machine, and CPG-bound oligonucleotide was removed from the column and placed in an Eppendorf tube (1.5 ml).

## 2. Radioactive Labelling of Oligonucleotide

To obtain  $^{35}\text{S}$ -labelled oligonucleotide, synthesis was carried out in two steps. The first 19 nucleotides of the sequence SEQ ID NO:1) from its 3'-end were assembled using the  $\beta$ -cyanoethylphosphoramidite approach (see, Beaucage in *Protocols for Oligonucleotides and Analogs* (Agrawal, ed.), Humana Press, (1993), pp. 33-61). The last six nucleotides were assembled using the H-phosphonate approach (see, Froehler in *Protocols for Oligonucleotides and Analogs* (Agrawal, ed.) Humana Press, 1993, pp.

63-80). Controlled pore glass (CPG) support-bound oligonucleotide (30 mg of CPG; approximately 1  $\mu$ M) containing five H-phosphonate linkage was oxidized with  $^{35}\text{S}_8$  (4 mCi, 1 Ci/mg, Amersham; 1 Ci = 37 GBq) in 60 ml carbon disulfide/pyridine/triethylamine (10:10:1). The oxidation reaction was performed at room temperature for 1 hr with occasional shaking. Then 2  $\mu$ l, 5  $\mu$ l, and 200  $\mu$ l of 5% cold sulfur ( $^{32}\text{S}_8$ ) in same solvent mixture was added every 30 min to complete the oxidation. The solution was removed and the CPG support was washed with carbon disulfide/pyridine/triethylamine (10:10:1) (3 x 500  $\mu$ l) and with acetonitrile (3 x 700  $\mu$ l). The product was deprotected in concentrated ammonium hydroxide (55°C, 14 hr) and evaporated. The resultant product was purified by polyacrylamide gel electrophoresis (20% polyacrylamide containing 7 M urea). The desired band was excised under UV shadowing and the PS-oligonucleotide was extracted from the gel and desalted with a Sep-Pak C18 cartridge (Waters) and Sephadex G-15 column. The yield was 20  $A_{260}$  units (600  $\mu$ g; specific activity, 1  $\mu$ Ci/ $\mu$ g).

To prepare  $^{35}\text{S}$ -labelled chimeric oligonucleotide, CPG-bound oligonucleotide was treated with a mixture of elemental  $^{35}\text{S}$  (4.5 mCi/mg atom, in 50  $\mu$ l of toluene; Amersham) in a solution of carbon disulfide, pyridine and triethylamine (200  $\mu$ l, 200  $\mu$ l and 4  $\mu$ l, respectively) at 25°C for 1 hr. 3H-1,2-benzodithiole-3-one-1,1-dioxide (1 ml, 2% in acetonitrile) was added, and the reaction mixture was allowed to remain at 25°C for

10 min. The supernatant was removed and the CPG-bound oligonucleotide was washed with  $\text{CH}_3\text{CN}$  (10 x 1 ml). After capping with acetic anhydride (300  $\mu\text{l}$ , tetrahydrofuran-lutidine-acetic anhydride, 8:1:1) and dimethylaminopyridine (300  $\mu\text{l}$ , 0.625% in pyridine), the  $^{35}\text{S}$ -CPG-bound oligonucleotide was washed with acetonitrile (10 x 1 ml) and packed in the column. For the next eight couplings, we used nucleoside  $\beta$ -cyanoethylphosphoramidite followed by oxidation with 3H-1,2-benzodithiole-3-one-1,1-dioxide. The last four couplings were carried out by using nucleoside methylphosphonamidite followed by oxidation with iodine reagent. The crude CPG-bound 25mer chimeric oligonucleotide was treated with concentrated ammonium hydroxide (28%, 3 ml) at 25°C for 2 hr. Evaporation on a Speed-Vac concentrator yielded a dried yellow pellet as crude  $^{35}\text{S}$ -labelled chimeric PS-oligonucleotide, which was immediately treated with a solution of ethylenediamine-ethanol-water (50:45:5, v/v/v/, 4 ml) for 4.5 hr at 25°C. Purification by PAGE (20% polyacrylamide, 7 M urea) gave pure  $^{35}\text{S}$ -labeled chimeric oligonucleotide as a white pellet (194  $A_{260}$  units, 155  $\mu\text{Ci}$ , 180  $\mu\text{Ci/mol}$ ). Other chemicals and reagents used in the present study were of the highest grade available.

### 3. Animals and Treatment

Male Sprague-Dawley rats (110  $\pm$  10 g, Harlan Laboratories, Indianapolis, IN) and male CD-/F2 mice (25  $\pm$  3 g, Charles River Laboratory, Wilmington, MA) were used in the study. The animals were fed with commercial diet and water *ad*

*libitum* for 1 week prior to the study.

Unlabelled and  $^{35}\text{S}$ -labelled oligonucleotides were dissolved in physiological saline (0.9% NaCl) in a concentration of 25 mg/ml, and were administered to the fasted animals via gavage at the designated dose (30-50 mg/kg for rats and 10 mg/kg for mice). Doses were based on the pretreatment body weight and rounded to the nearest 0.01 ml. After dosing, each animal was placed in a metabolism cage and fed with commercial diet and water *ad libitum*. Total voided urine was collected and each metabolism cage was then washed following the collection intervals. Total excreted feces was collected from each animal at various timepoints, and feces samples were homogenized prior to quantitation of radioactivity. Blood samples were collected in heparinized tubes from animals at the various timepoints. Plasma was separated by centrifugation. Animals were euthanized by exsanguination under sodium pentobarbital anesthesia at various times (i.e., 1, 3, 6, 12, 24, and 48 hr; 3 animals/time point). Following euthanasia, the tissues were collected from each animal. All tissues/organs were trimmed of extraneous fat or connective tissue, emptied and cleaned of all contents, individually weighed, and the weights recorded prior to homogenization.

To quantitate the total absorption of the hybrid oligonucleotide, two additional groups of animals (3 per group) for each test oligonucleotide were treated using the same

procedure as above. Animals were killed at 6 or 12 hr post dosing, and the gastrointestinal tract was then removed. Radioactivities in the gastrointestinal tract, feces, urine, plasma, and the remainder of the body were determined separately. Total recovery of radioactivity was also determined to be  $95 \pm 6\%$ . The percentage of the absorbed hybrid oligonucleotide-derived radioactivity was determined by the following calculation:

$$\frac{\text{(total radioactivity in the remainder of the body + total radioactivity in urine)}}{\text{(total radioactivity in the gastrointestinal tract, feces, urine, plasma, and the remainder of the body)}} \times 100\%$$

#### 4. Total Radioactivity Measurements

The total radioactivities in tissues and body fluids were determined by liquid scintillation spectrometry (LS 6000TA, Beckman, Irvine, CA). In brief, biological fluids (plasma, 50-100  $\mu$ l; urine, 50-100  $\mu$ l) were mixed with 6 ml scintillation solvent (Budget-Solve, RPI, Mt. Prospect, IL) to determine total radioactivity. Feces were ground and weighed prior to being homogenized in a 9-fold volume of 0.9% NaCl saline. An aliquot of the homogenate (100  $\mu$ l) was mixed with solubilizer (TS-2, RPI, Mt. Prospect, IL) and then with scintillation solvent (6 ml) to permit quantitation of total radioactivity.

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Following their removal, tissues were immediately blotted on Whatman No. 1 filter paper and weighed prior to being homogenized in 0.9% NaCl saline (3-5 ml per gram of wet weight). The resulting homogenate (100  $\mu$ l) was mixed with solubilizer (TS-2, RPI, Mt. Prospect, IL) and then with scintillation solvent (6 ml) to determine total radioactivity. The volume of 0.9% NaCl saline added to each tissue sample was recorded. The homogenized tissues/organs were kept frozen at  $\leq -70^{\circ}\text{C}$  until the use for further analysis.

#### 5. HPLC Analysis

The radioactivity in urine was analyzed by paired-ion HPLC using a modification of the method described essentially by Sands et al. (*Mol. Pharm.* (1994) 45:932-943). Urine samples were centrifuged and passed through a 0.2- $\mu\text{m}$  Acro filter (Gelman, Ann Arbor, MI) prior to analysis. Hybrid oligonucleotide and metabolites in plasma samples were extracted using the above methods in sample preparation for PAGE. A Microsorb MV-C4 column (Rainin Instruments, Woburn, MA) was employed in HPLC using a Hewlett Packard 1050 HPLC with a quaternary pump for gradient making. Mobile phase included two buffers; Buffer A was 5 mM-A reagent (Waters Co., Bedford, MA) in water and Buffer B was 4:1 (v/v) Acetonitrile (Fisher)/water. The column was eluted at a flow rate of 1.5 ml/min, using the following gradient: (1) 0-4 min, 0% buffer B; (2) 4-15 min 0-35% Buffer B; and (3) 15-70 min 35%-80% Buffer B. The column was equilibrated with Buffer A for at least

30 min prior to the next run. By using a RediFrac fraction collector (Pharmacia LKB Biotechnology, Piscataway, NJ), 1-min fractions (1.5 ml) were collected into 7-ml scintillation vials and mixed with 5 ml scintillation solvent to determine radioactivity in each fraction.

6. PAGE and Autoradiography

Plasma and tissue homogenates were incubated with proteinase K (2 mg/ml) in extraction buffer (0.5% SDS/10 mM NaCl/20 mM Tris-HCl, pH 7.6/10 mM EDTA) for 1 hr at 60°C. The samples were then extracted twice with phenol/chloroform (1:1, v/v) and once with chloroform. After ethanol precipitation, the extracts were analyzed by electrophoresis in 20% polyacrylamide gels containing 7 M urea. Urine samples were filtered, desalted and then analyzed by polyacrylamide gel electrophoresis (PAGE). The gels were fixed in 10% acetic acid/10% methanol solution and then dried before autoradiography.

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain, using no more than routine experimentation, numerous equivalents to the specific substances and procedures described herein. Such equivalents are considered to be within the scope of this invention, and are covered by the following claims.